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Visualizing Intermediates on the Reaction Pathway of the R61 D-ala-D-ala Peptidase

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Introduction: Antibiotic resistance is an ever-increasing problem in treating bacterial infections. This pressure has lead to the use of structural approaches to assist in the development of new antibiotics since knowledge of the three-dimensional topology of a drug-binding site can aid in the design of new antibacterial agents. We are studying a 37.5kDa enzyme that is a target for β-lactam antibiotics (penicillins and cephalosporins), the D-alanyl-D-alanine carboxypeptidase/transpeptidases (DD-peptidase) from *Streptomyces* sp. R61. This enzyme is a member of a group of proteins called penicillin-binding proteins (PBPs) that function via an active site serine residue. Its normal function is to serve as a peptidase in the final stages of bacterial cell wall biosynthesis when the peptide cross-links are formed in the bacterial peptidoglycan strands to give the cell wall its structural integrity. β-Lactam antibiotics mimic the normal peptide substrates of the PBPs but with a key difference. When the active site serine hydrolyzes the β-lactam bond, a long-lived acyl-enzyme intermediate is formed that prevents the PBP from carrying out its catalytic reaction in cell wall synthesis in growing bacteria. The bacteria subsequently lyse and die, and the infection is cured.

Extensive crystallographic studies have been performed on the R61 DD-peptidase in complex with a variety of β-lactam inhibitors. However, despite the wealth of experimental data detailing the interactions between the enzyme and inhibitors, little is known about how the enzyme interacts with its natural substrate. Inhibitors inhibit enzymes because they interact differently than the natural substrate. The fact that this enzyme remains active in crystalline form has hampered crystallographic studies of interactions between substrates and the enzyme since the reaction is too fast to observe crystallographically. Recently, Dr. R.F. Pratt and coworkers at Wesleyan University synthesized a novel tetrapeptide substrate that is the exact terminal peptide portion of *S.* R61 peptidoglycan. This molecule has been shown kinetically to be the most specific substrate for the R61 DD-peptidase studied to date. Modifying our experimental conditions and using this substrate allowed us to trap three distinct intermediates in the catalytic pathway of this enzyme: a non-covalent Henri-Michaelis (ES) complex, a covalent acyl-enzyme intermediate (ES*), and a non-covalent enzyme-products (EPs) complex.

$$\mathbf{E + S \leftrightarrows ES \rightarrow ES^{*} \rightarrow EPs \leftrightarrows E + Ps}$$

Results: We had previously determined the structures of the native enzyme to 1.0Å resolution (R=0.10 with R_{free}=0.13) and the non-covalent Henri-Michaelis (ES) complex (R=0.15 with R_{free}=0.18, 1.9Å resolution). This was possible because we had observed previously that prolonged exposure to trace amounts of formaldehyde in the crystallization buffer inactivated enzyme crystals by cross-linking three highly conserved active site residues: K65, H108, and Y159. By soaking formaldehyde-inactivated enzyme crystals with the tetrapeptide substrate, we had been able to trap the substrate as a non-covalent complex with the DD-peptidase (ES). In experiments performed during this cycle, we successfully trapped a peptidyl acyl-enzyme intermediate (ES*) and the noncovalent enzyme-products complex (EPs) and determined their structures. ES* was trapped using a phosphonate analog of the tetrapeptide substrate prepared by Dr. Pratt and his group. Soaking active enzyme crystals with this molecule yielded a structure of a peptidyl acyl-enzyme intermediate, the first such complex observed with any DD-peptidase (R=0.11 with R_{free}=0.13, 1.2Å resolution). Owing to the tetrahedral geometry of the phosphorus atom, this structure also represents a tetrahedral transition-state. To isolate the last intermediate, the EPs complex, active enzyme crystals were soaked with tetrapeptide and a 40-fold molar excess of D-alanine, one of the products of the carboxypeptidase reaction. In these experiments, we were able to trap the non-covalent enzyme-products complex (R=0.11 with R_{free}=0.15, 1.2Å resolution). Thus, using synchrotron radiation and manipulating our peptidase crystals, we were successful in determining the structures of the kinetic intermediates ES, ES^{*}, and EPs in the reaction pathway.